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Abstract: Acute lymphoblastic leukemia (ALL) accounts for 25% of pediatric malignancies. Of interest, the incidence of ALL is observed 20% higher in males relative to females. The mechanism behind the phenomenon of sex-specific differences is presently not understood. Employing genome-wide genetic aberration screening in 19 ALL samples, one of the most recurrent lesions identified was monoallelic deletion of the 5' region of SLX4IP. We characterized this deletion by conventional molecular genetic techniques and analyzed its interrelationships with biological and clinical characteristics using specimens and data from 993 pediatric patients enrolled into trial AIEOP-BFM ALL 2000. Deletion of SLX4IP was detected in 30% of patients. Breakpoints within SLX4IP were defined to recurrent positions and revealed junctions with typical characteristics of illegitimate V(D)J-mediated recombination. In initial and validation analyses, SLX4IP deletions were significantly associated with male gender and ETV6/RUNX1-rearranged ALL (both overall $P < 0.0001$). For mechanistic validation, a second recurrent deletion affecting TAL1 and caused by the same molecular mechanism was analyzed in 1149 T-cell ALL patients. Validating a differential role by sex of illegitimate V(D)J-mediated recombination at the TAL1 locus, 128 out of 1149 T-cell ALL samples bore a deletion and males were significantly more often affected ($P = 0.002$). The repeatedly detected association of SLX4IP deletion with male sex and the extension of the sex bias to deletion of the TAL1 locus suggest that differential illegitimate V(D)J-mediated recombination events at specific loci may contribute to the consistent observation of higher incidence rates of childhood ALL in boys compared with girls.

DOI: https://doi.org/10.1093/hmg/ddt447

Human Molecular Genetics, 2014, Vol. 23, No. 3 ***590–601***
doi:10.1093/hmg/ddt447
Advance Access published on September 17, 2013

Frequent and sex-biased deletion of *SLX4IP* by illegitimate V(D)J-mediated recombination in childhood acute lymphoblastic leukemia

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Received May 25, 2013; Revised and Accepted September 12, 2013

Acute lymphoblastic leukemia (ALL) accounts for ~25% of pediatric malignancies. Of interest, the incidence of ALL is observed ~20% higher in males relative to females. The mechanism behind the phenomenon of sex-specific differences is presently not understood. Employing genome-wide genetic aberration screening in 19 ALL samples, one of the most recurrent lesions identified was monoallelic deletion of the 5′ region of *SLX4IP*. We characterized this deletion by conventional molecular genetic techniques and analyzed its interrelationships with biological and clinical characteristics using specimens and data from 993 pediatric patients enrolled into trial AIEOP-BFM ALL 2000. Deletion of *SLX4IP* was detected in ~30% of patients. Breakpoints within *SLX4IP* were defined to recurrent positions and revealed junctions with typical characteristics of illegitimate V(D)J-mediated recombination. In initial and validation analyses, *SLX4IP* deletions were significantly associated with male gender and *ETV6/RUNX1*-rearranged ALL (both overall $P < 0.0001$). For mechanistic validation, a second recurrent deletion affecting *TAL1* and caused by the same molecular mechanism was analyzed in 1149 T-cell ALL patients. Validating a differential role by sex of illegitimate V(D)J-mediated recombination at

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the *TAL1* locus, 128 out of 1149 T-cell ALL samples bore a deletion and males were significantly more often affected ($P = 0.002$). The repeatedly detected association of *SLX4IP* deletion with male sex and the extension of the sex bias to deletion of the *TAL1* locus suggest that differential illegitimate V(D)J-mediated recombination events at specific loci may contribute to the consistent observation of higher incidence rates of childhood ALL in boys compared with girls.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy (1–3). Despite intensive research efforts, the causes of childhood ALL remain largely unknown. Sex bias—characterized by a 20% higher incidence in males relative to females—is consistently observed in childhood ALL (2–4). In addition, male sex is associated with worse treatment response and outcome (5,6). Therefore, a better understanding of this phenomenon could directly enhance our understanding of disease etiology and improve therapeutic approaches to ALL.

From a genetic perspective, ALL is characterized by recurrent numeric and/or structural somatic aberrations (7). Hyperdiploidy or the cryptic chromosomal translocation t(12;21)—leading to an *ETV6/RUNX1* gene fusion—are detectable in 20–25% of cases each, making them the most common genetic subtypes of childhood ALL (7,8). Important for pathomechanistic insights, the molecular features associated with the breakpoints of structural genetic aberrations in ALL are multifold and include, for example, an open chromatin context during gene transcription, Alu and other repeat sequences, or illegitimate V(D)J-mediated recombination (7).

V(D)J recombination is a physiological process by which segments (variable (V), diversity (D), joining (J)) of immunoglobulin (Ig) or T-cell receptor (TCR) genes are rearranged and lead to great diversity of the Ig/TCR repertoire. This process is mediated by lymphocyte-specific endonucleases (RAG1, RAG2) which cut the regional V(D)J genes at flanking recombination signal sequences (RSS) consisting of specific highly conserved heptamer and nonamer sequences with an unconserved spacer (12 or 23 nucleotides) in-between (9,10). Subsequently, the coding segments are joined using the classical non-homologous end-joining (NHEJ) pathway. Paradoxically, RAG proteins can recognize a variety of slightly modified RSS sequences increasing the chance of erroneously targeting RSS-like sequences ('cryptic RSS') elsewhere in the genome. Several chromosomal translocations between Ig/TCR loci and proto-oncogenes as well as deletions of non-antigen receptor loci in lymphoid malignancies are thought to be generated by such illegitimate V(D)J-mediated recombination events (11,12). Recently, interstitial deletion of the *B-cell translocation gene 1* (*BTG1*) in 9% of precursor B-cell ALL has been attributed to this mechanism (13). Also, subtypes of *CDKN2A* and *IKZF1* deletions as well as all *TAL1* deletions have been associated with off-target action of the RAG complex (12,14–16).

In the present study, we describe a highly frequent deletion in *SLX4IP*—a gene encoding a currently uncharacterized DNA repair-related protein—by illegitimate V(D)J-mediated recombination (17). *SLX4IP* deletion has previously been reported in childhood ALL, but at a far lower frequency (18,19). We correlate this finding to demographic and clinical characteristics in a large cohort of children with ALL and describe its specific association with *ETV6/RUNX1*-rearranged ALL and male sex.

RESULTS

In our initial screen for recurrent genetic aberrations in childhood ALL employing comparative genomic hybridization (CGH) analysis, 5 out of 19 samples—making it one of the most common observations—harbored a monoallelic deletion encompassing the first two exons of the *SLX4IP* gene with tight breakpoint clustering to a defined position (HG18, chromosome 20: ~10 363 654–10 404 199 bp; Fig. 1A). Sequencing analysis in the five deletion-positive samples revealed patient-specific breakpoints within only a few nucleotides of each other—confirming site specificity of the recombination event—and demonstrated near perfect matches to 5′ and 3′ heptamer sequences (consensus 5′-CACAGTG), non-templated 'N' nucleotides and 'nibbling away' of nucleotides at the junction points as hallmarks of illegitimate V(D)J-mediated recombination (Fig. 1B–D) (11,12,14,16,20). No convincing AT-rich nonamer sequences (consensus 5′-ACAAAAACC) were found at 12 or 23 nucleotides distance of the heptamers. As all five patients displaying the *SLX4IP* deletion at initial diagnosis relapsed, we next analyzed ALL samples obtained at disease recurrence for maintenance of aberrations during leukemic evolution and detected the same patient-specific deletion breakpoints in all samples (Fig. 1D).

As the above analyses were performed on selected patients, we next aimed at a reliable assessment of the *SLX4IP* deletion frequency in a cohort of 512 patients enrolled into AIEOP-BFM ALL 2000 (Cohort 1, Table 1, Supplementary Material, Fig. S1) (5,6). Employing a PCR assay, the deletion could be detected in 164 children (32.0%). When analyzing the association of *SLX4IP* deletion with clinical characteristics, the most significant positive interrelationships were observed for male sex ($P < 0.001$) and *ETV6/RUNX1*-rearranged ALL ($P < 0.001$) (Table 1). In an analysis stratified by *ETV6/RUNX1* translocation status, the male sex bias stayed significant in translocation positive ($P < 0.001$) as well as negative patients ($P < 0.001$) (Tables 2 and 3). No association of *SLX4IP* deletion with treatment outcome was detected (Supplementary Material, Fig. S2).

To validate the observed interrelationships, we next screened an independent cohort of 232 patients with *ETV6/RUNX1*-rearranged ALL from AIEOP-BFM ALL 2000 (Cohort 2) and identified 145 (62.5%) deletion-positive samples. In these analyses, both the association of *SLX4IP* deletion with male sex ($P = 0.049$) and its high incidence in *ETV6/RUNX1*-rearranged ALL could be confirmed (Table 3, Supplementary Material, Table S1). In a second validation step, we screened 249 leukemic *ETV6/RUNX1*-negative samples from AIEOP-BFM ALL 2000 patients enrolled in the years 2001 and 2002 (Cohort 3) and, again, found a significant positive association of *SLX4IP*

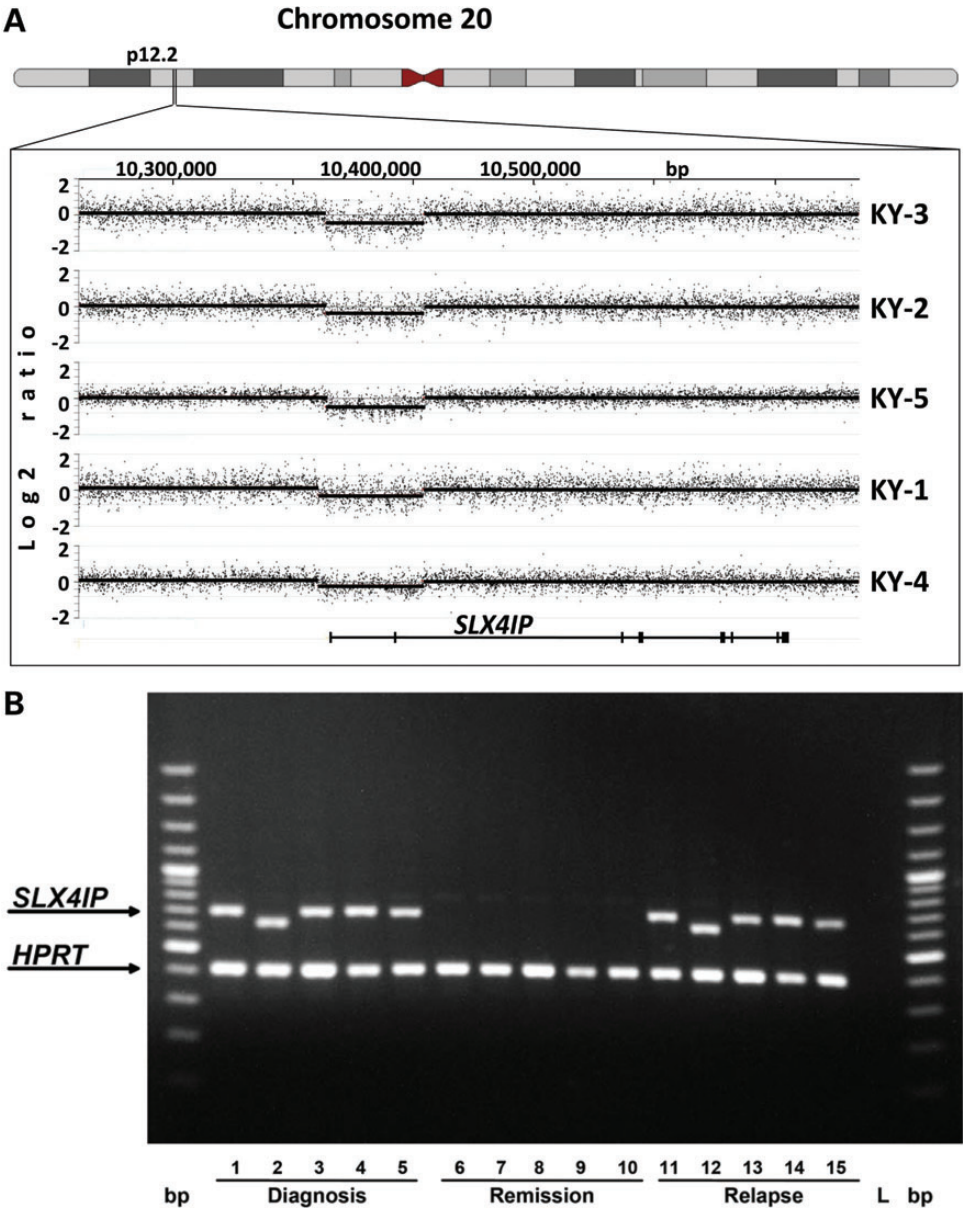


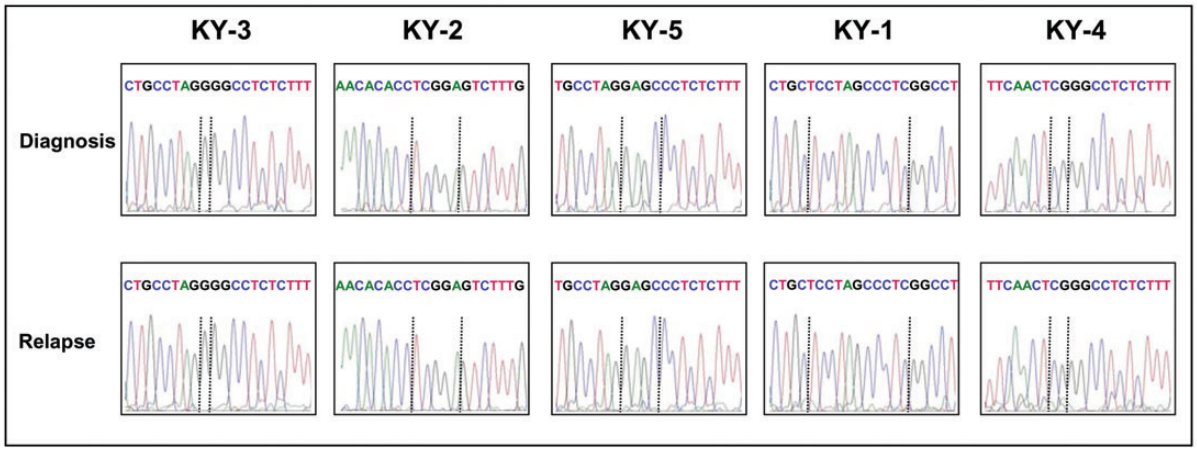
Figure 1. Deletion of the first two exons of *SLX4IP* on chromosome 20 in five *ETV6/RUNX1*-negative ALL samples. (**A**) Custom made NimbleGen fine-tiling CGH-array illustrating a uniform deletion at diagnosis. (**B**) PCR product of the same samples showing the *SLX4IP* deletion at diagnosis and relapse. No PCR products regarding *SLX4IP* deletion are discovered in remission (germline). Patient samples are depicted in the following order: KY-3: lines 1, 6 and 11; KY-2: lines 2, 7 and 12; KY-5: lines 3, 8 and 13; KY-1: lines 4, 9 and 14; KY-4: lines 5, 10 and 15. bp = 100 base pair DNA ladder. L = negative control. *HPRT* exons 7 and 8 were used as positive control. (**C**) The *SLX4IP* deletion junction sequence is displayed and remains unchanged from diagnosis to relapse. Dotted lines illustrate breakpoints at the 5′ and 3′ end of the deletion and N-nucleotides are allocated in-between. (**D**) The *SLX4IP* deletion junction sequence at diagnosis and relapse is shown in alignment with wild-type sequence. Signs of illegitimate V(D)J-mediated recombination are displayed. The pseudo-heptamer as part of the pseudo-RSS is depicted as black box. The potential pseudo-nonamer sequence is underlined, separated either by a 12-RSS or 23-RSS.

deletion status with male sex ($P = 0.029$) (Table 3, Supplementary Material, Table S2).

To consolidate our findings on *SLX4IP* deletion characteristics at the nucleotide level which were based on five samples only, we continued by sequencing 38 additional leukemic patient DNA samples and detected site-specific *SLX4IP* breakpoint junctions with typical characteristics of illegitimate V(D)J-mediated recombination in all specimens (Supplementary Material, Table S3). This suggests that—similar to T-cell ALL with its high frequency of illegitimate V(D)J-mediated recombination events—precursor B-cell ALL bears site-specific recombination events with non-dispersed breakpoints in association with cryptic RSS at a previously underestimated high frequency (>25%). Of importance, ~50% of samples exhibited signs of oligoclonality, indicating that different *SLX4IP*-deleted subclones existed in parallel.

Recently, it was demonstrated that subclones in ALL have variegated genetics associated with a dynamic clonal architecture in the lead-up to a diagnosis and in relapse (21). To characterize the behavior of the *SLX4IP* deletion during leukemic

C



D

5' wildtype AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGCCTAGCACCGTCAGTTACCATGATACGTTATTTGCTAATGAGAGTCATTGGGGTCCC

Patient No.	Timepoint	Deletion junction sequence upstream of 5' breakpoint	Insertion (*N-Nucleotides*)	Deletion junction sequence downstream of 3' breakpoint
KY-3	Diagnosis	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGCCTAG	*G*	GGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
	Relapse	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGCCTAG	*G*	GGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
KY-2	Diagnosis	AAAACACACC	*TCGGA*	GTCTTTGCAT
	Relapse	AAAACACACC	*TCGGA*	GTCTTTGCAT
KY-5	Diagnosis	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGCCTAG	*GAGC*	CCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
	Relapse	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGCCTAG	*GAGC*	CCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
KY-1	Diagnosis	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGC	*TCCTAGCCCTC*	GGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
	Relapse	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACTGC	*TCCTAGCCCTC*	GGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
KY-4	Diagnosis	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACT	*CG*	GGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT
	Relapse	AAAACACACCACAGAAAAGCGTTTTTTTTTCTCTCGGTCAGTTCAACT	*CG*	GGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT

3' wildtype CCTGGAGGTAGCTTCAGGTTGCCCATGGCCCTGAGGCAGCTTCACTGTGGGCCTCTCTTTGAGAAGTGGGATTCTGGTCTTTGCAT

Figure 1. Continued

evolution, we extended our above described preliminary analyses of initial diagnosis and relapse samples to a total of 22 pairs (Fig. 1; Supplementary Material, Table S4). The deletion was confirmed at relapse in 21 paired samples by PCR. Sequence analysis of the respective breakpoints showed the same monoclonal sequences in nine patients indicating stability of *SLX4IP* deletions during disease progression. The remaining samples either developed heterogeneity of *SLX4IP* breakpoints at relapse subsequent to monoclonal sequences ($n = 1$) or already showed an oligoclonal pattern at initial diagnosis ($n = 11$) (Supplementary Material, Fig. S3). Backtracking of *SLX4IP* deletions to birth in seven *ETV6/RUNX1*-positive patients using material derived from Guthrie cards did not yield any positive results suggesting that deletion of *SLX4IP* is a secondary event occurring after birth (Supplementary Material, Fig. S4). Although backtracking of *SLX4IP* deletions may have been hampered by the sensitivity of the detection method, a secondary nature of the deletions is supported by the observation that in monozygotic twins with *ETV6/RUNX1*-positive ALL and identical translocation breakpoints, the *SLX4IP* deletion was detectable in one diagnostic leukemic sample only while the second twin was negative (Supplementary Material, Fig. S5). None of 134 available matching bone marrow remission samples from Cohort 1 exhibited the *SLX4IP* deletion as well as none of 145 peripheral blood samples derived from healthy blood donors.

To gain information on functional consequences of *SLX4IP* deletion, we next compared its expression in 60 diagnostic ALL specimens carrying the deletion with 60 deletion-negative samples (Supplementary Material, Table S5). We did not find significant differences by *SLX4IP* deletion status ($P = 0.793$), but *ETV6/RUNX1*-positive samples demonstrated higher *SLX4IP* expression compared with *ETV6/RUNX1* negatives (Fig. 2, $P < 0.001$). Next, we investigated a potential effect of *SLX4IP* deletion on the expression of neighboring genes (*MKKS* and *JAG1*, Supplementary Material, Table S5 and S6) (22,23). While no differential expression of the *MKKS* gene was seen ($P = 0.826$), patients carrying a *SLX4IP* deletion demonstrated significantly higher expression of *JAG1* ($P < 0.001$). Also here, we performed stratified analysis by *ETV6/RUNX1* status and observed significantly higher expression of *JAG1* in *SLX4IP*-deleted samples in the *ETV6/RUNX1*-negative subgroup ($P = 0.011$), whereas positive patients demonstrated uniformly high *JAG1* expression with no discernable differences according to *SLX4IP* deletion status ($P = 0.999$) (Fig. 2).

Table 1. Clinical characteristics of 512 patients with ALL from trial AIEOP-BFM ALL 2000 (Cohort 1) by *SLX4IP* deletion status

	Patients positive for *SLX4IP* deletion ($n = 164$) *n* (%)	Patients negative for *SLX4IP* deletion ($n = 348$) *n* (%)	*P*-value[d]
Gender			
Male	124 (75.6)	181 (52.0)	
Female	40 (24.4)	167 (48.0)	<0.001
Age at diagnosis (years)			
<1	0 (0.0)	0 (0.0)	
1–<5	79 (48.2)	161 (46.3)	
5–<10	49 (29.9)	90 (25.9)	
10–<15	23 (14.0)	73 (21.0)	
≥15	13 (7.9)	24 (6.9)	0.286
Initial WBC[a] (μl^{-1})			
<10 000	80 (48.8)	156 (44.8)	
10 000–<50 000	56 (34.1)	111 (31.9)	
50 000	16 (9.8)	46 (13.2)	
≥100 000	12 (7.3)	35 (10.1)	0.463
Immunophenotype			
Pre-B	142 (86.6)	260 (74.7)	
Pro-B	5 (3.0)	14 (4.0)	
T-cell	17 (10.4)	72 (20.7)	0.011
Other/unknown	0 (0.0)	2 (0.6)	
DNA index[b]			
<1.16	117 (71.3)	208 (59.8)	
≥1.16	8 (4.9)	64 (18.4)	<0.001
Unknown	39 (23.8)	76 (21.8)	
ETV6/RUNX1			
Positive	71 (43.3)	39 (11.2)	
Negative	82 (50.0)	283 (81.3)	<0.001
Unknown	11 (6.7)	26 (7.5)	
BCR/ABL			
Positive	5 (3.0)	8 (2.3)	
Negative	158 (96.4)	332 (95.4)	0.765
Unknown	1 (0.6)	8 (2.3)	
MLL/AF4			
Positive	0 (0.0)	0 (0.0)	
Negative	152 (92.7)	320 (92.0)	NA[e]
Unknown	12 (7.3)	28 (8.0)	
Prednisone response[c]			
Good	151 (92.1)	294 (84.5)	
Poor	12 (7.3)	52 (14.9)	0.015
Unknown	1 (0.6)	2 (0.6)	
Risk group			
Standard	61 (37.2)	112 (32.2)	
Intermediate	83 (50.6)	169 (48.6)	
High	20 (12.2)	67 (19.3)	0.123

[a]WBC, white blood cell count at diagnosis.
[b]Ratio of DNA content of leukemic G^0/G^1 cells to normal diploid lymphocytes.
[c]Good: <1000 leukemic blood blasts/μl on treatment Day 8; poor: ≥1000 μl^{-1}.
[d]χ^2- or Fisher's exact test.
[e]NA, not applicable.

To find out whether an increased male susceptibility to illegitimate V(D)J-mediated recombination was restricted to the *SLX4IP* locus or could be extended to other loci, we finally analyzed illegitimate V(D)J-mediated deletions of *TAL1* in a cohort 1149 T-cell ALL patients from trials AIEOP-BFM ALL 2000 and ALL IC-BFM 2002 (Cohort 4). We were able to identify 128 (11.1%) *TAL1*-deleted patients with 110 (85.9%) out of them being male. Similar to *SLX4IP*, this distribution resulted in a significant positive association of *TAL1* deletion status with male sex ($P = 0.002$) (Table 3). Thus, an increased male susceptibility to the event of illegitimate V(D)J-mediated recombination could also be observed at the *TAL1* locus. In additional analyses employing multiplex ligation-dependent probe amplification (MPLA) data on *IKZF1* and *BTG1* deletions which were previously described as being V(D)J-mediated we could not detect a sex-biased distribution of deletions (Supplementary Material, Table S7) (13,24,25).

DISCUSSION

Here, we describe a site-specific deletion within the 5′ region of the *SLX4IP* locus in ∼30% of childhood ALL in general and >60% of *ETV6/RUNX1*-rearranged ALL, making the deletion of *SLX4IP* one of the most common aberrations in childhood ALL described so far. As *SLX4IP* deletions were previously only described at far lower frequencies in array-based genome-wide analyses of representative large cohorts (18,19), our observation of *SLX4IP* deletion at a much higher percentage by PCR analysis implies that the subclonal architecture of ALL in association with methodological sensitivity thresholds introduces bias in frequency estimates of genetic aberrations by conventional SNP array or CGH analyses. Most likely, a larger proportion of *SLX4IP* deletions detected in our study occurred at later stages of leukemic evolution secondary to *ETV6/RUNX1* rearrangements and were, therefore, only present in a fraction of leukemic cells. These subclonal levels would have been missed by array techniques requiring presence of a lesion in a majority of analyzed cells for reliable detection. The particular sensitivity of the *SLX4IP* locus to secondary recombination events is further exemplified by the fact that breakpoint sequencing revealed oligoclonality in nearly half of the *SLX4IP* deletion-positive samples analyzed at initial diagnosis of ALL.

The described deletion in the 5′ region of *SLX4IP* differs from other recurrently detectable genetic aberrations of comparable frequencies in childhood ALL in the way that it occurs in a site-specific fashion and consistently demonstrates features of a single causal mechanism—illegitimate V(D)J-mediated recombination. The repeatedly detected association of *SLX4IP* deletion with male sex in our study and the extension of the sex bias to deletion of the *TAL1* locus imply that differential susceptibility by sex to illegitimate V(D)J-mediated recombination at specific loci may contribute to the consistent observation of higher incidence rates of childhood ALL in boys compared with girls. We were not able to extend our findings to two additional loci recurrently displaying features of illegitimate V(D)J-mediated recombination at their breakpoints—*IKZF1* and *BTG1* (13,14). This suggests that our thoroughly validated observation of sex-specific acquisition of *SLX4IP* and *TAL1* deletions by illegitimate V(D)J-mediated recombination cannot be generalized to all loci demonstrating susceptibility to illegitimate V(D)J-mediated recombination. The mechanism underlying these locus-specific findings remains to be clarified. Potential explanations could include locus-specific differences, variation in sex-specific susceptibility to illegitimate V(D)J-mediated recombination for biological subgroups of ALL, or *SLX4IP* and *TAL1* deletions occurring with equal frequencies in males and females, but underlying sex-specific differences in selection processes during leukemia development.

Sex differences exist in a variety of diseases including, besides others, autoimmune disorders and infectious diseases, and are

Table 2. Clinical characteristics of Cohort 1 according to *SLX4IP* deletion status in *ETV6/RUNX1*-positive and -negative ALL patients treated on AIEOP-BFM ALL 2000

	ETV6/RUNX1-positive patients ($n = 110$)		*P*-value[d]	*ETV6/RUNX1*-negative patients ($n = 365$)		*P*-value[d]
	Positive for *SLX4IP* deletion ($n = 71$) *n* (%)	Negative for *SLX4IP* deletion ($n = 39$) *n* (%)		Positive for *SLX4IP* deletion ($n = 82$) *n* (%)	Negative for *SLX4IP* deletion ($n = 283$) *n* (%)	
Gender						
Male	47 (66.2)	12 (30.8)		68 (82.9)	154 (54.4)	
Female	24 (33.8)	27 (69.2)	<0.001	14 (17.1)	129 (45.6)	<0.001
Age at diagnosis (years)						
<1	0 (1.4)	0 (0.0)		0 (0.0)	0 (0.0)	
1–<5	42 (59.2)	22 (56.4)		30 (36.6)	126 (44.5)	
5–<10	23 (32.4)	14 (35.9)		24 (29.3)	70 (24.7)	
10–<15	4 (5.6)	3 (7.7)		19 (23.2)	64 (22.6)	
≥15	2 (2.8)	0 (0.0)	0.708	9 (11.0)	23 (8.1)	0.564
Initial WBC[a] (μl^{-1})						
<10 000	40 (56.3)	23(59.0)		36 (43.9)	118 (41.7)	
10 000–<50 000	21 (29.6)	9 (23.1)		32 (39.0)	95 (33.6)	
50 000	7 (9.9)	6 (15.4)		5 (6.1)	39 (13.8)	
≥100 000	3 (4.2)	1 (2.6)	0.738	9 (11.0)	31 (11.0)	0.292
Immunophenotype						
Pre-B	70 (98.6)	38 (97.4)		63 (76.8)	202 (71.4)	
Pro-B	1 (1.4)	1 (2.6)		4 (4.9)	11 (3.9)	
T-cell	0 (0.0)	0 (0.0)	NA[e]	15 (18.3)	68 (24.0)	0.516
Other/unknown	0 (0.0)	0 (0.0)		0 (0.0)	2 (0.7)	
DNA index[b]						
<1.16	49 (69.0)	30 (76.9)		60 (73.2)	167 (59.0)	
≥1.16	4 (5.6)	1 (2.6)	0.647	3 (3.6)	57 (20.1)	<0.001
Unknown	18 (25.4)	8 (20.5)		19 (23.2)	59 (20.9)	
Prednisone response[c]						
Good	69 (97.2)	39 (100.0)		73 (89.0)	230 (81.3)	
Poor	2 (2.8)	0 (0.0)	0.538	8 (9.6)	52 (18.4)	0.067
Unknown	0 (0.0)	0 (0.0)		1 (1.2)	1 (0.4)	
Risk group						
Standard	35 (49.3)	28 (71.8)		21 (25.6)	78 (27.6)	
Intermediate	34 (47.9)	11 (28.2)		46 (56.1)	142 (50.2)	
High	2 (2.8)	0 (0.0)	0.058	15 (18.3)	63 (22.3)	0.609

[a]WBC, white blood cell count at diagnosis.
[b]Ratio of DNA content of leukemic G^0/G^1 cells to normal diploid lymphocytes.
[c]Good: <1000 leukemic blood blasts/μl on treatment Day 8; poor: ≥1000 μl^{-1}.
[d]χ^2- or Fisher's exact test.
[e]NA, not applicable.

largely believed to represent differential effects of sex-specific hormone action even in prepubertal children (26,27). For the hematopoietic system, it was demonstrated that 17β-estradiol treatment can modify the differentiation, proliferation and survival of early B-cell precursors with a direct influence on Ig gene rearrangements (28). How these observations could mechanistically relate to sex-specific differences in illegitimate V(D)J-mediated recombination in childhood ALL is currently only subject to speculation, but it may well be that differences in endogenous sex hormone exposure could play a modifying role here. Although sex hormone levels between boys and girls already differ during infancy, it is of interest that sex bias in childhood ALL becomes most obvious in children during puberty when hormonal differences become more profound (27). That nuclear receptors themselves are implicated in the mechanism of genomic aberrations was recently demonstrated by an intriguing observation demonstrating that binding of the androgen receptor to intronic regions introduces inter- and intrachromosomal interactions, leading to double-strand breaks and ligation by the NHEJ pathway (29). Future mechanistic studies taking into account this available information may shed some light on the complex interplay of factors with potential involvement in sex-biased genetic aberrations in childhood ALL.

If the high frequency of the deletion indicates a pathomechanistic role for *SLX4IP* in ALL leukemogenesis and confers a selective advantage to the affected leukemic clone or simply reflects an increased locus-specific susceptibility to recombination events without 'leukemogenic' consequences remains to be evaluated in further detail. Nevertheless, we found that *SLX4IP* expression did not differ between leukemic samples carrying the deletion and those not, indicating that loss at *SLX4IP* seems to be predominantly monoallelic as demonstrated by array CGH and does not lead to haploinsufficiency at the transcriptional level. In contrast, we could demonstrate higher expression of the *SLX4IP* neighboring gene *JAG1* in *SLX4IP*-deleted patients. Overexpression of *JAG1*—a WNT-dependent Notch signaling activator with important functions in stem and progenitor cell homeostasis—has been previously reported in acute myeloid leukemia as well as *ETV6/RUNX1*-positive ALL (22,23). Since ETV6—through a consensus sequence on the promoter—may function as a transcriptional repressor on *JAG1*, the decrease of ETV6 activity as a consequence of the *ETV6/RUNX1* translocation

Table 3. Gender distribution by *SLX4IP* or *TAL1* deletion status in four childhood ALL cohorts from trials AIEOP-BFM ALL 2000 and ALL IC-BFM 2002

	Cohort 1[a] ($n = 512$) All patients			*ETV6/RUNX1*-positive ($n = 110$)			*ETV6/RUNX1*-negative ($n = 365$)		
	SLX4IP deletion-positive ($n = 164$) *n* (%)	*SLX4IP* deletion-negative ($n = 348$) *n* (%)	*P*-value[b]	*SLX4IP* deletion-positive ($n = 71$) *n* (%)	*SLX4IP* deletion-negative ($n = 39$) *n* (%)	*P*-value[b]	*SLX4IP* deletion-positive ($n = 82$) *n* (%)	*SLX4IP* deletion-negative ($n = 283$) *n* (%)	*P*-value[b]
Gender									
m[c]	124 (75.6)	181 (52.0)		47 (66.2)	12 (30.8)		68 (82.9)	154 (54.4)	
f[c]	40 (24.4)	167 (48.0)	<0.001	24 (33.8)	27 (69.2)	<0.001	14 (17.1)	129 (45.6)	<0.001
	Cohort 2[d] ($n = 232$)			Cohort 3[e] ($n = 249$)			Cohort 4[f] ($n = 1149$)		
	SLX4IP deletion-positive ($n = 145$) *n* (%)	*SLX4IP* deletion-negative ($n = 87$) *n* (%)	*P*-value[b]	*SLX4IP* deletion-positive ($n = 25$) *n* (%)	*SLX4IP* deletion-negative ($n = 224$) *n* (%)	*P*-value[b]	*TAL1* deletion-positive T-cell ALL ($n = 128$) *n* (%)	*TAL1* deletion-negative T-cell ALL ($n = 1021$) *n* (%)	*P*-value[b]
Gender									
m[c]	81 (55.9)	37 (42.5)		19 (76.0)	119 (53.1)		110 (85.9)	750 (73.5)	
f[c]	64 (44.1)	50 (57.5)	0.049	6 (24.0)	105 (46.9)	0.029	18 (14.1)	271 (26.5)	0.002

[a]Analysis in Cohort 1 included stratification regarding *ETV6/RUNX1* translocation and DNA index.
[b]χ^2- or Fisher's exact test.
[c]m, male; f, female.
[d]Cohort 2 comprised only *ETV6/RUNX1* translocation-positive patients.
[e]Cohort 3 comprised only *ETV6/RUNX1* translocation-negative patients.
[f]Cohort 4 consisted of 1149 children with T-cell ALL who have been investigated for *TAL1* deletions and were recruited from the German ALL-BFM 2000 and additional national trial groups from Austria, Italy, Czech Republic and Israel.

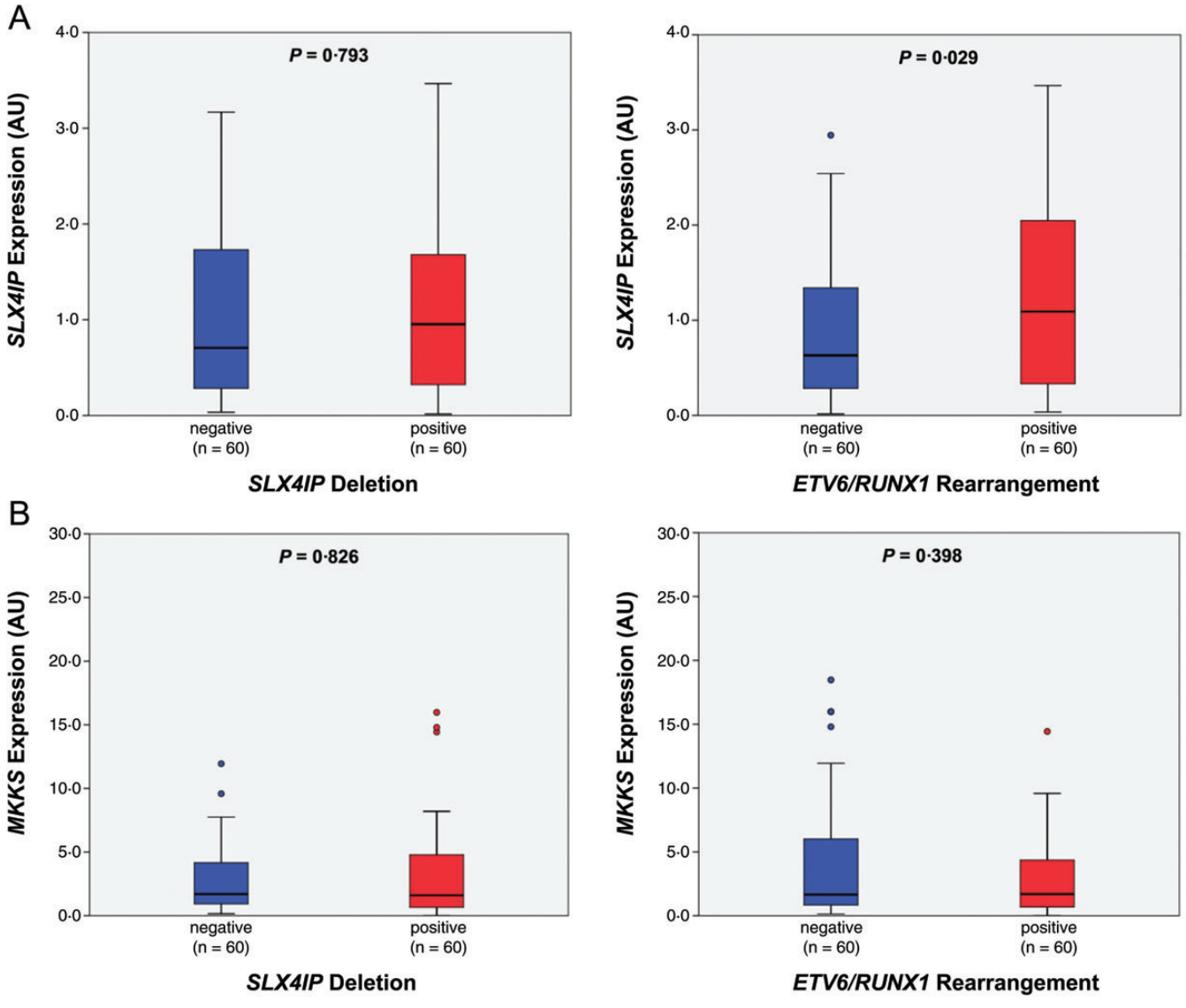


Figure 2. Expression of SLX4IP, MKKS and JAG1 by *SLX4IP* deletion and *ETV6/RUNX1* rearrangement status. SLX4IP, MKKS and JAG1 expression in leukemic bone marrow cells at diagnosis was measured by real-time quantitative polymerase chain reaction after reverse transcription. The horizontal line in each box indicates the median. The top and bottom of each box indicate the first and third quartiles, respectively, and the tails of the boxes extend to the most extreme values not considered to be outliers. *P*-values of the Mann–Whitney *U*-test are depicted. (**A**) No difference in SLX4IP expression is observed in *SLX4IP* deletion-positive and -negative patients. Patients with *ETV6/RUNX1* rearrangement have a slightly higher SLX4IP expression than *ETV6/RUNX1*-negative patients. (**B**) MKKS expression is the same in *SLX4IP* deletion positive and negative as well as in *ETV6/RUNX1* rearrangement-positive and -negative patients. (**C**) JAG1 expression is depicted in all examined patients ($N = 319$). *SLX4IP* deletion-positive patients as well as *ETV6/RUNX1* rearrangement-positive patients have significantly higher JAG1 expression in comparison to deletion- or rearrangement-negative patients. (**D**) JAG1 expression after stratification according to *ETV6/RUNX1* rearrangement: *SLX4IP* deletion-positive ALL samples do express significantly higher JAG1 levels compared with *ETV6/RUNX1* rearrangement-negative samples, whereas *ETV6/RUNX1* rearrangement-positives all demonstrate high JAG1 expression independent of *SLX4IP* deletion status. AU, arbitrary units.

was suggested to be responsible for the increase of *JAG1* transcription in *ETV6/RUNX1*-positive ALL (23). However, although we detected differential *JAG1* expression by *SLX4IP* deletion status in the overall patient group and in the *ETV6/RUNX1*-negative subgroup, this effect could not be observed in *ETV6/RUNX1*-positive patients. This observation contradicts a selective advantage of a leukemic cell acquiring a *SLX4IP* deletion by dysregulation of *JAG1* and supports a model where higher regional transcriptional activity at *SLX4IP* and the neighboring *JAG1* is common to *ETV6/RUNX1*-rearranged and a subgroup of alike other ALLs which predisposes to *SLX4IP* deletion through an open chromatin context. Thus, *SLX4IP* deletion may act as a surrogate of a local transcriptional profile common to *ETV6/RUNX1*-positive ALL and a group of *ETV6/RUNX1*-like ALL with a similarly good prognosis.

In conclusion, the here reported increased susceptibility of males to illegitimate V(D)J-mediated recombination at *SLX4IP* and *TAL1* may serve as an important etiological hint to the currently only poorly understood sex bias in childhood ALL and, at the same time, may serve the hypothesis of sex-specific differences in recombination frequencies being associated with higher male incidences in a majority of hematological neoplasms and their partly less favorable treatment outcome.

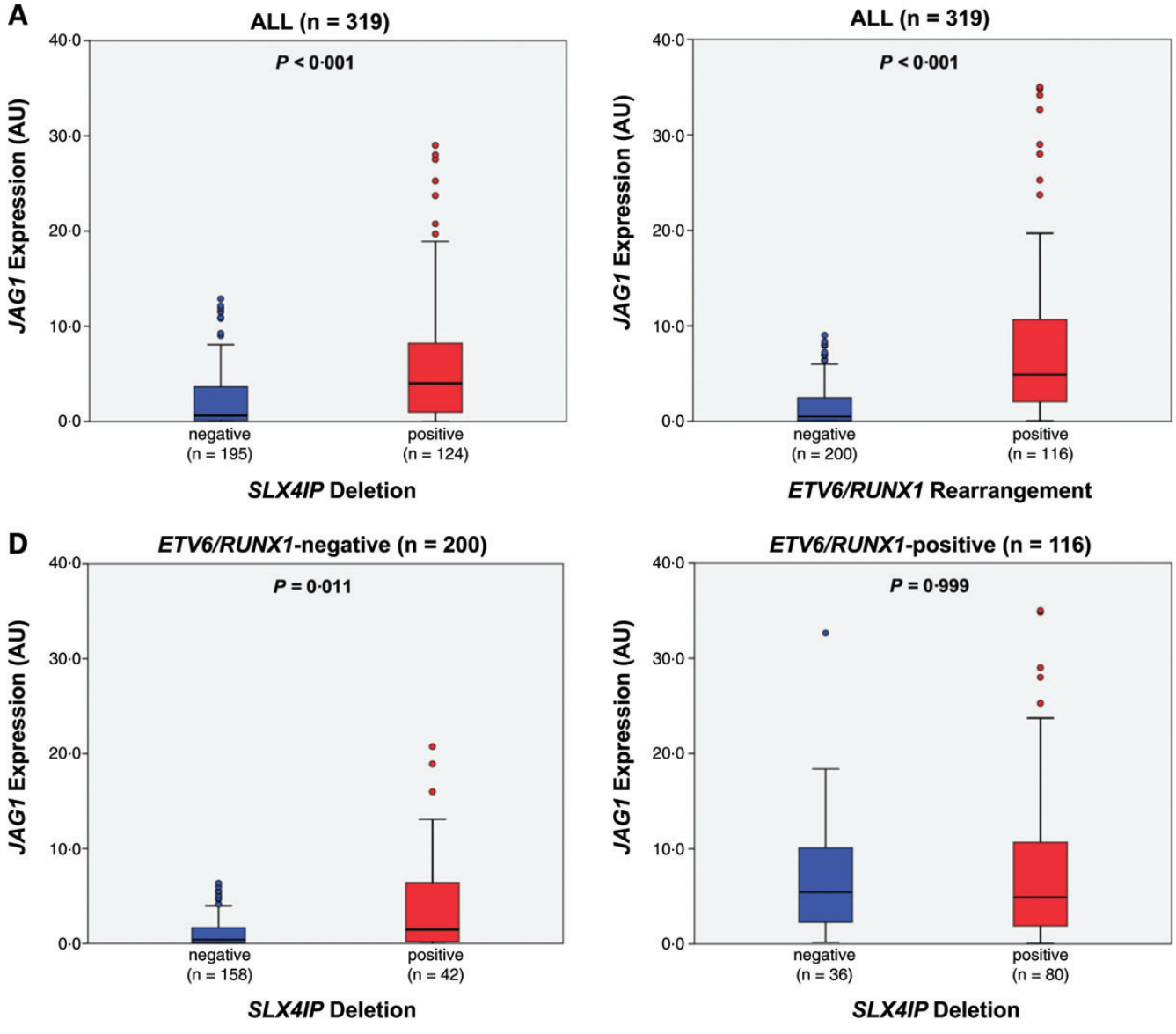


Figure 2. Continued

MATERIALS AND METHODS

Study individuals

Patients from Austria, Germany, Italy and Switzerland were enrolled in multicenter trial AIEOP-BFM ALL 2000 on treatment of childhood ALL. Diagnosis, characterization and treatment of ALL were performed as previously described (see also Supplementary Material) (5,6,30–35).

German patients of AIEOP-BFM ALL 2000 were included for *SLX4IP* deletion screening and sequencing, *TAL1* deletion analysis as well as gene expression analysis, Austrian and Italian patients were only included in *TAL1* deletion analysis. Patients from Czech Republic and Israel were treated in multicenter trial ALL IC-BFM 2002. Czech patients were included in backtracking experiments and subjected to *TAL1* deletion analysis, Israeli patients only employed for *TAL1* analysis. German patients with recurrent ALL were enrolled in relapse trials ALL-Rez BFM 95/96 and ALL-Rez BFM 2002 and included in *SLX4IP* sequencing analyses. Outcome analysis was only performed for German patients treated on AIEOP-BFM ALL 2000. All trials are described in detail elsewhere and were approved by the respective institutional review boards (Hannover Medical School, Hannover; 2nd Faculty of Medicine, Charles University Prague; Charité University Medicine, Berlin) (5,6,36–38). Informed consent for the use of specimen for research was obtained from all study individuals, parents or legal guardians.

Samples, cell and DNA isolation

Bone marrow samples were obtained at initial diagnosis, treatment Days 15, 33, 52, 78 and at relapse. Mononuclear cells were isolated by Ficoll-Paque gradient centrifugation

(Pharmacia, Freiburg, Germany) from bone marrow samples followed by extraction of high-molecular-weight DNA according to standardized protocols using either the Puregene DNA isolation system (Gentra Systems, Minneapolis, MN, USA) or Qiagen DNA Blood Kits (Qiagen, Hilden, Germany). Quality and quantity of genomic DNA was determined by spectrophotometry. DNA yielded from bone marrow aspirates collected in remission served for germline *SLX4IP* deletion screening. An anonymized cohort of blood donors served as healthy control group.

Comparative genomic hybridization analysis

Nineteen diagnostic DNA samples (blast content >80%) from German AIEOP-BFM ALL 2000 patients (Supplementary Material, Table S8) who later relapsed and matching remission bone marrows were hybridized to high-resolution custom-made fine-tiling CGH arrays covering 62 loci comprising 27 210 197 bp (Supplementary Material, Table S9). Loci selection was based on own preliminary experiments employing 100K Affymetrix arrays (Affymetrix, Santa Clara, CA, USA) and publications reporting genomic aberrations in ALL (18).

UCSC Human genome build March 2006 (HG18) was used as a reference. The designed oligonucleotide array comprised 385 000 probes (probe length 50–75mer) and was manufactured, hybridized and scanned by imaGenes GmbH (NimbleGen service: ROCHE NimbleGen, Reykjavik, Iceland). Repeat regions were excluded and oligonucleotide spacing was <71 bp on average (Supplementary Material, Table S9). Data were analyzed using SignalMap Version 1.9.0.03 (NimbleGen Systems, Inc., Madison, WI, USA).

SLX4IP and *TAL1* deletion screening and genomic sequencing, MLPA

SLX4IP deletion breakpoints were amplified by genomic PCR using primers (5′ → 3′) forward: gataattcacccggcatttccccatc and reverse: atgcccccgaggcctctctacaaact (39). Product length was ∼705 bp when *SLX4IP* deletion was present; sequencing primers (5′ → 3′) were forward: tgttcaaacatggctatttttatt and reverse: cctgtctatgagactgccaaa. For *TAL1* deletion analysis, diagnostic leukemic DNA samples were screened using the BIOMED-1 primer set (40).

Amplification of *SLX4IP* deletion breakpoints was performed by genomic PCR (forward primer (5′–3′): gataattcacccggcatttccccatc and reverse primer (5′–3′): atgcccccgaggcctctctacaaact) (39). Fifty nanograms of leukemic blast DNA or human control DNA were amplified using the Qiagen Mutation Detect PCR Kit (Qiagen). As a positive control, a fragment encompassing *HPRT* exons 7 and 8 was amplified using the following primers: forward (5′–3′): gtaatattttgtaattaacagcttgctgg and reverse (5′–3′): tcagtctggtcaaatgacgaggtgc (product length 423 bp). PCR conditions were: 95°C for 5 min followed by 35 cycles of 95°C for 30 s and 61°C for 1 min and 72°C for 3 min. PCR products were analyzed on a multicapillary electrophoresis system (QIAxcel, Qiagen). Before direct sequencing, PCR products were run on a 1.0% agarose gel and purified using the Qiagen gel extraction Kit (Qiagen). Product length was ∼705 bp when *SLX4IP* deletion was present. The nucleotide sequences of both strands of the PCR products were directly determined using an automated fluorescent sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer Corporation, Wellesley, MA, USA). Primer sequences used for sequencing were the following: forward (5′–3′): tgttcaaacatggctatttttatt and reverse (5′–3′): cctgtctatgagactgccaaa. When distinct breakpoints with non-ambiguous sequencing results continuing behind the breakpoint junction in forward and reverse sequencing reactions were observed, monoclonality of *SLX4IP* deletion was assumed (Fig. 1C). In the case of non-ambiguous sequencing results before the breakpoint junction (in forward and reverse reactions) with sudden indistinct sequencing signature or with one major-sequence as well as another upcoming minor sequence signature behind the typical breakpoint junction, oligoclonality was hypothesized (Supplementary Material, Tables S3 and S4 and Fig. S3). In these cases, true breakpoints might deviate by 1–2 nucleotides from those depicted in Supplementary Material, Tables S5 and S6, due to ambiguous breakpoint junction assignment.

Primer design in the BIOMED-1 approach for the *TAL1* deletion was restricted to the breakpoint in the *SIL* gene and the two most frequent breakpoints in the *TAL1* gene, type 1 and type 2 (taldb1 and taldb2), covering at least 95% of all known *TAL1* deletions. PCR products obtained were further examined by heteroduplex or gene scanning analyses to discriminate between amplifications derived from monoclonal or polyclonal lymphoblastic cell populations and junctional regions of clonal PCR products were sequenced.

MLPA was performed as described previously (25).

Deletion backtracking in Guthrie cards and *ETV6/RUNX1* genomic breakpoint cloning

For backtracking of *SLX4IP* deletions to the time of birth, genomic DNA from Guthrie cards was isolated using the InstaGene Dry Blood Kit (Bio-Rad, Hercules, CA, USA). Three separate sections (1/8) of one Guthrie card were used in PCR reactions. For *SLX4IP* deletion screening, specific primers were designed. The amplified products were analyzed by automated chip-based microcapillary electrophoresis on an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Assay specificity was determined using serial dilutions of individual diagnostic DNA in healthy donor DNA. Guthrie card PCRs were performed in a separate room in a separate PCR box. For *SLX4IP* deletion screening in Guthrie cards specific primers were designed: forward (5′–3′): agaaaacacaccacagaaaagc and reverse (5′–3′): gggaggtgaggagccactat. PCR conditions were: initial denaturation at 95°C for 10 min; 35 cycles at 94°C for 30 s, 58.5°C for 30 s, 72°C for 30 s, final extension 72°C for 7 min. *ETV6/RUNX1* genomic breakpoints in monozygotic twins were cloned as described previously (41).

RNA isolation, reverse transcription and gene expression analysis by real-time quantitative PCR

Total RNA was isolated with Triazol reagent (Invitrogen, Paisley, UK) and subsequently passed over a Qiagen RNeasy column (Qiagen) for removal of small fragments. Total RNA was quantified and validated for integrity using the Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Real-time quantitative polymerase chain reaction (RQ-PCR) analysis was performed after

random hexamer priming and MuLV reverse transcription (Fermentas, Hanover, MD, USA) to generate cDNA. PCR was carried out on an Applied Biosystems Model 7900 HT Sequence Detector (Darmstadt, Germany) using the QuantiTect SYBR Green PCR kit (Qiagen) as described in the manufacturer's instructions. The expression level of the *SDHA* and *ABL1* were used to normalize for differences in input cDNA. QuantiTect Primer Assays were used to measure mRNA abundances of the *SLX4IP*, *MKKS* and *JAG1* genes (Qiagen). Melting curve analyses were performed to verify the amplification specificity. Each sample was tested in duplicate. The expression ratio was calculated as $2n$, where n was the threshold cycle (C_T) value difference normalized by the CT difference of a calibrator sample.

For *SLX4IP* and *MKKS* expression analysis 120 German patients from trial AIEOP-BFM ALL 2000 with known *SLX4IP* deletion status and RNA availability were selected. Selection criteria were as following: half of the 120 patient samples were either *SLX4IP* deletion-negative ($n = 60$) or positive ($n = 60$). Within each of the two subgroups, half of the patients were *ETV6/RUNX1* translocation-negative ($n = 30$) or positive ($n = 30$). Exclusion criteria comprised: *BCR/ABL1* or *MLL* rearrangement, T-ALL and hyperdiploidy (Supplementary Material, Table S5). For *JAG1* expression analysis, 319 German patients from trial AIEOP-BFM ALL 2000 with known *SLX4IP* deletion status and RNA availability were selected (Supplementary Material, Table S6).

Statistical analysis

Differences in the distribution of categorical variables among patient subsets were analyzed using Fisher's exact or χ^2-test. Comparisons of gene expression levels between groups were performed by Mann–Whitney *U*-test. Event-free survival was defined as the time from diagnosis to the date of last follow-up in complete remission or to the first event. Events were resistance to therapy (non-response), relapse, secondary neoplasm or death from any cause. Failure to achieve remission due to early death or non-response was considered as event at time zero. Patients lost to follow-up were censored at the time of their withdrawal. The Kaplan–Meier method was used to estimate survival rates, differences were compared with the two-sided log-rank test (42,43). Cumulative incidence functions for competing events were constructed by the method of Kalbfleisch and Prentice, and were compared employing the Gray's test (44,45). Computations were performed using SPSS Version 17.0 (SPSS, Armonk, NY, USA) and SAS Version 9.1 (SAS, Cary, NC, USA) statistical programs.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We are indebted to all participants and personnel involved in the clinical trials associated with this study.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Deutsche José Carreras Leukämie-Stiftung, the Madeleine Schickedanz Kinderkrebs-Stiftung, the Deutsche Krebshilfe, the Israel Cancer Association, Fondazione Tettamanti (Monza), Fondazione Citta della Speranza, Fondazione Cariparo (Padova) and St. Anna Kinderkrebsforschung Austria.

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